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ArcR Modulates Biofilm Formation in the Dental Plaque Colonizer *Streptococcus gordonii*

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SUMMARY

Biofilm formation and cell-cell sensing by the pioneer dental plaque coloniser *Streptococcus gordonii* is dependent upon arginine. This study aimed to identify genetic factors linking arginine-dependent responses and biofilm formation in *S. gordonii*. Isogenic mutants disrupted in genes required for biosynthesis or catabolism of arginine, or for arginine-dependent gene regulation, were screened for their ability to form biofilms in a static culture model. Biofilm formation by a knockout mutant of *arcR*, encoding an arginine-dependent regulator of transcription, was reduced to <50% that of the wild-type whereas other strains were unaffected. Complementation of *S. gordonii* Δ*arcR* with a plasmid-borne copy of *arcR* restored the ability to develop biofilms. By DNA microarray analysis, 25 genes were differentially regulated in *S. gordonii* Δ*arcR* compared with wild-type under arginine-replete conditions including 8 genes encoding components of phosphotransferase systems for sugar uptake. By contrast, disruption of *argR* or *ahrC* genes, which encode paralogous arginine-dependent regulators, each resulted in significant changes in the expression of more than 100 genes. Disruption of a gene encoding a putative extracellular protein that was strongly regulated in *S. gordonii* Δ*arcR* had a minor impact on biofilm formation. We hypothesise that genes regulated by ArcR form a critical pathway linking arginine sensing to biofilm formation in *S. gordonii*. Further elucidation of this pathway may provide new targets for the control of dental plaque formation by inhibiting biofilm formation by a key pioneer coloniser of tooth surfaces.

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INTRODUCTION

The formation of dental plaque is initiated by the attachment of pioneer colonisers to the tooth surface.[1] Oral streptococci including *S. gordonii*, *S. sanguinis*, *S. oralis* and *S. mitis* are particularly well adapted for the initial colonisation of tooth surfaces since they produce a multitude of cell surface adhesin proteins and glycoproteins that recognise host receptors in the salivary pellicle.[2-4] Many of these adhesins also contribute to the subsequent development of dental plaque by mediating cell-cell binding, known as coaggregation, with other oral bacteria.[5-7]

Oral streptococci are considered to be opportunistic pathogens since they can enter the bloodstream and are among the leading causes of the rare, but life-threatening, disease infective endocarditis.[8] However, in mature dental plaque there is evidence that oral streptococci protect against dental caries. Thus, some species produce arginine deiminase, which generates ammonia and neutralises plaque acid leading to shifts in the microbiome towards health.[9-11] Arginine deiminase also directly influences other oral bacteria by acting as an interspecies signalling molecule. For example, the arginine deiminase of *Streptococcus cristatus* is sensed by the periodontal pathobiont *Porphyromonas gingivalis* and leads to down-regulation of virulence gene expression.[12] The uptake of arginine by oral streptococci such as *S. gordonii* occurs through an arginine-ornithine antiporter, ArcD, and ornithine released into the growth medium can be utilised by other species such as *Fusobacterium nucleatum*.[13] However, at high concentrations (≥50 mM), arginine can disrupt coaggregation between *S. gordonii* and *F. nucleatum* by inhibiting the *F. nucleatum* adhesin RadD.[14,15] High concentrations of arginine also disrupt multispecies oral microbial biofilms or *S. gordonii* monospecies biofilms.[16-19]

There is evidence that oral streptococci such as *S. gordonii* use arginine as a key signal for growth processes and biofilm formation. *Streptococcus* and related genera including *Enterococcus*, *Lactococcus* and *Lactobacillus* possess between 2 and 4 copies of ArgR family arginine-sensing transcriptional regulators, enabling close co-ordination of arginine biosynthesis, catabolism and transport.[19] The *S. gordonii* genome, for example, encodes 3 different ArgR family regulators designated ArgR, AhrC and ArcR. Shifting *S. gordonii* cells from arginine-replete medium to medium lacking arginine results in changes in expression of >450 genes, nearly one quarter of the entire genome.[19] Arginine sensing and biofilm formation pathways are triggered by intermicrobial interactions. For example, interactions with *Candida albicans* lead to down-regulation of *S. gordonii arcA* and *arcB* encoding components of the arginine deiminase system, and up-regulation of the biofilm-associated operon *fruRBA*.[20] Coaggregation of *S. gordonii* with *A. oris* resulted in the co-ordinated down-regulation of 9 arginine biosynthesis genes and up-regulation of the biofilm-promoting *bfb* locus.[21] Coaggregation with *A. oris* also enabled the growth of *S. gordonii* in arginine-restricted medium. Therefore, it is possible that *S. gordonii* arginine-dependent regulators are employed to sense cell-cell interactions and respond by initiating growth and/or biofilm formation.

On the basis of the above observations, we hypothesised that one or more genes involved in arginine regulation and/or metabolism is linked to biofilm formation in oral streptococci. Since *S. gordonii* has been well-characterised in terms of responses to arginine, we used this species as a model to explore the genetic basis of the link between arginine and biofilm formation by oral bacteria. Initially, a molecular genetic approach was employed to screen for components of arginine pathways (regulation, biosynthesis, or catabolism) that are linked to biofilm formation. To obtain further insights into arginine-mediated gene regulation, we characterised the regulons of the three *S. gordonii* ArgR homologues, ArcR, ArgR and AhrC.

METHODS

Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table S1. *S. gordonii* was routinely cultured in THYE medium containing 30 g L^{-1} Bacto™ Todd Hewitt Broth (Becton Dickinson, Oxford, UK) and 5 g L^{-1} Yeast Extract (Melford Laboratories Ltd, Ipswich, UK) or on solidified THYE containing 15 g L^{-1} Bacto-agar (Becton Dickinson). Cells were cultured in candle jars without shaking for 24-48 h at 37^{o}C. For biofilm assays, *S. gordonii* was cultured in TYEG medium containing 10 g L^{-1} Bacto Tryptone, 5 g L^{-1} Yeast Extract, 3 g L^{-1} K_2HPO_4 and 2 g L^{-1} D-glucose, adjusted to pH 7.5 before autoclaving. *E. coli* was cultured in Luria–Bertani (LB) medium at 37^{o}C, 250 rpm or on LB medium solidified by the addition of 15 g L^{-1} Bacto-agar. When required antibiotics were included in growth media at the following concentrations: erythromycin 2 µg mL^{-1}, spectinomycin 100 µg mL^{-1}, kanamycin 250 µg mL^{-1}.

Genetic manipulation of S. gordonii

Routine genetic manipulations were conducted in accordance with standard protocols.[22] Previously constructed gene knockout mutants are described in Table S1. Disruption of SGO_RS04150 (here designated '*earA*') was performed using PCR overlap extension mutagenesis as described by Jakubovics et al[19]. Briefly, flanking regions of the *earA* gene were PCR amplified using primers *earA* F1 ovex and *earA* R1 kan ovex to generate an 869 bp product in the 5' region of *earA* and kan F1 ovex and kan R1 ovex to generate a 903 bp product in the 3' end of *earA* (Table S2). The *aphA3* cassette (910 bp) was amplified from plasmid pSF151 with primers aphA3F2/R2.[19] The PCR products were stitched together in an overlap extension PCR reaction. The resulting product was cleaned and used for transformation of *S. gordonii* DL1. Successful disruption and replacement of *earA* gene with the *aphA3* cassette was confirmed by DNA sequencing.

To produce a genetic complementation strain (*S. gordonii arcR*$_{Comp}$), plasmid p*arcR*$_{Comp}$ was generated using the In-Fusion HD PCR ligation cloning kit (Clontech, Mountain View, CA). A 181 bp region of the synthetic CP25 promoter was amplified from plasmid pCM18,[23] using primers CP25F and CP25R. Primers pPE1010F and pPE1010R were used to amplify a 5,652 bp fragment of vector pPE1010.[24] Primers *arcR*CompF and *arcR*CompR, containing 15bp regions of overlap with pPE1010, were utilised to amplify a 494 bp fragment containing the *arcR* gene from *S. gordonii* chromosomal

DNA. The In-Fusion HD PCR ligation cloning kit was employed to fuse the CP25 promoter and *arcR* gene into the pPE1010 vector. The integrity of plasmid p*arcR*$_{Comp}$ was confirmed by sequencing, and p*arcR*$_{Comp}$ was used for transformation of *S. gordonii* Δ*arcR::aad9* to generate *S. gordonii arcR*$_{Comp}$.

Crystal violet biofilm assay

Biofilms for crystal violet assays were cultured on the surface of Cellstar® 96-well microtiter plates in TYEG medium without shaking, aerobically for 18 h at 37°C (Greiner Bio-one, Stonehouse, UK). The biomass was measured as described by Shields et al[25] Biofilms were submerged in 100 µL of 0.5% (w/v) crystal violet. After incubation at 20°C for 15 min, wells were washed three times in 200 µL of PBS, air-dried, and residual crystal violet was dissolved with 100 µL of 7% (v/v) acetic acid and quantified by measuring A_{562}. All experiments were performed three times independently. Statistical significance of differences between mutants and wild-type *S. gordonii* was assessed by ANOVA with Dunnett's post-hoc test, and $p<0.05$ was considered significant.

Fluorescent staining and Imaging

Biofilms for visualization experiments were cultured on sterile glass coverslips incubated in wells of 6-well tissue culture dishes. Following growth, biofilms were rinsed with PBS and incubated with Live/Dead BacLight stain (Molecular Probes) for 15 min at 20°C. For confocal laser scanning microscopy (CLSM), stained coverslips were rinsed with PBS and inverted onto a PBS-filled Gene frame (25 µL, 1.0 x 1.0 cm, Thermo Fischer Scientific) secured on a microscope slide. Imaging was performed using a Nikon A1R confocal laser scanning microscope fitted with CFI PLAN APO VC objective (Nikon 60x/1.40 Oil). Images were captured with NIS-Elements C (v4.4, Nikon) software and processed using Imaris (v8.2, Bitplane) software. Biovolume quantification of Z-stacks was conducted using Volocity software (v6.3, PerkinElmer, UK), set to identify objects ≥1 µm^2 as *S. gordonii* cells. At least three Z-stacks (image size 1024 x 1024) from three different fields for view was analysed for each strain. The data were analysed from three independent experiments. Statistical significance of differences between biofilm biovolume was assessed using ANOVA with Tukey's post-hoc test.

Growth in chemically defined medium and RNA extraction

For gene regulation analysis, chemically-defined FMC medium [19] was used with 0.5 mM L-arginine HCl (Sigma-Aldrich, Dorset, UK) included or omitted as appropriate. Briefly, *S. gordonii* was cultured in FMC medium aerobically at 37°C for 18 h. Cells were harvested, washed with fresh FMC and resuspended in 20 mL FMC medium. Cultures were incubated at 37°C until an OD_{600} of 0.3-0.4 was reached. At this point, cultures were split into 5 mL aliquots, and cells were harvested and resuspended in 5 mL of either fresh arginine-replete FMC, or FMC without L-arginine. Cells were cultured at 37°C for a further 30 min. To extract RNA, 1 volume of RNALater was added, cultures were vortex-mixed and incubated for 5 min at 20°C. Cells were harvested and, after discarding the supernatant, pellets were stored for up to 5 days at -80°C. Cell pellets were thawed on ice and resuspended in 100 µl spheroplasting buffer [26% (w/v) raffinose, 10 mM $MgCl_2$, 20 mM Tris-HCl, pH 6.8] containing 0.1 mg/ml chloramphenicol or spectinomycin. Mutanolysin (500 U mL^{-1}) was

added to cells and incubated at 37°C for 5 min before addition of 350 µl RNAWiz solution (Life Technologies). The mixture was vortexed vigorously for 15 s and RNA was extracted using the Ambion RiboPure Bacteria RNA Purification kit (Life Technologies, Carlsbad, California, US) in accordance with the manufacturer's recommendations.

Gene expression analysis by RT-qPCR

For RT-qPCR, samples were reverse transcribed with QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA). Reactions were performed according to manufacturer's instructions, with the modification that 3 µg/mL random hexamer primers (Bioline, London, UK) were used in place of the QuantiTect oligo-dT primers. The cDNA was cleaned and used as template in RT-qPCR experiments with the SensiMix SYBR No-ROX kit (Bioline) with the following reaction conditions: 1. 95°C for 10 min, 2. 95°C for 15 s, 3. 60°C for 1 min, 4. plate read, 5. repeat from step 2 a further 39 times, 6. melting curve from 55-90°C, read every 1°C, hold for 5 s. All samples were normalised against the 16S rDNA gene. Primer sets for this and for *argC, argG, pyrA$_b$, arcA, arcB, arcD* and *amyB* genes have previously been published.[19] Other primers are described in Table S2. Standard curves, melting curves and agarose gel electrophoresis analysis of the cDNA, were routinely included to validate the RT-qPCR experiments.

Microarray analysis

Microarray analysis was performed as described by Jakubovics et al[19] using a previously designed microarray containing 2,051 probes for *S. gordonii* genes (GEO accession GPL17786). Samples of RNA from four independent experiments per strain/growth condition were sent to the Genomics and Microarray Facility, Birmingham University, UK for reverse transcription, labelling and hybridisation. The microarray series for *S. gordonii* DL1 in high or no arginine was previously deposited as GEO accession GSE51346. Other data series for arginine-dependent regulator mutants in high or no arginine were deposited in GEO with the following accession numbers: *S. gordonii* Δ*arcR*, GSE101509; *S. gordonii* Δ*argR*, GSE101506; *S. gordonii* Δ*ahrC*, GSE101507.

Data were analysed using Agilent GeneSpring GX software. Probe expression data was quantile normalised to enable unbiased comparisons between samples. To assess the relatedness of samples, principal component analysis (PCA) was carried out using the normalised data. Outliers identified in the PCA analysis were removed before proceeding to significance analysis. A moderated t-test was used to determine statistics for each probe. The resulting *p*-values were then adjusted using the Benjamini-Hochberg multiple testing correction procedure.[26] For each comparison, probes with a corrected *p*-value of ≤0.05 and with fold change numerically greater than 2 were considered to be differentially expressed between conditions. Rank product analysis was carried out using the RP method implemented in the R package 'RankProd'.[27,28] This method applies a non-parametric statistical test, based on rank ordering of genes according to fold changes, to detect genes that are consistently upregulated or downregulated in replicated experiments. The heatmap was produced using the R package ComplexHeatmap.[29]

RESULTS

Effects of disrupting arginine metabolism or regulation genes on S. gordonii *biofilm formation*

To investigate the genetic basis for the previously observed links between arginine sensing and biofilm formation in *S. gordonii*, a range of mutants lacking genes involved in arginine-dependent regulation, arginine catabolism or biosynthesis were screened for their ability to form biofilms in a high-throughput static 96-well microplate system. Biofilms were cultured for 24 h in TYEG medium, washed and stained with crystal violet to quantify the biofilm biomass (Figure 1). Biofilm formation was not significantly different from wild-type levels in any of the single mutants screened with the exception of *S. gordonii* Δ*arcR*, which formed approximately 50% less biofilm than *S. gordonii* DL1. This was not due to a defect in planktonic growth as the growth yield, measured as the OD_{600} of the well prior to washing and staining, was almost identical between *S. gordonii* DL1 and Δ*arcR* (data not shown). It was consistently observed that biofilms formed by *S. gordonii* Δ*arcR* appeared similar to those produced by the wild-type until they were agitated, indicating that cells were loosely attached. The *arcR* gene encodes an arginine-dependent regulator of the ArgR family. To assess whether ArcR acts in concert with other ArgR-family regulators to control biofilm formation, the *arcR* mutation was introduced into *S. gordonii* Δ*argR* and Δ*ahrC* backgrounds. In each case, biofilm formation by the double mutants containing an *arcR* knockout was similar to that of the Δ*arcR* single mutant, and approximately 50% reduced compared with wild-type. By contrast, an *S. gordonii* Δ*argR* Δ*ahrC* double mutant was not impaired in biofilm formation (Figure 1). Therefore, it appears that ArcR and not ArgR or AhrC is required for efficient biofilm formation in *S. gordonii*.

Visualisation of biofilms and genetic complementation of S. gordonii *Δ*arcR

Analysis of biofilms by CLSM with BacLight LIVE:DEAD staining revealed clear differences between biofilms formed by *S. gordonii* DL1 and *S. gordonii* Δ*arcR* (Figure 2). Biofilms formed by the wild-type were confluent and relatively smooth, and approximately 10-20 µm thick throughout. By contrast, *S. gordonii* Δ*arcR* biofilms were more heterogenous, with clumps of cells up to 40 µm thick and patches of surface that were not covered at all. To confirm that the observed biofilm defects were due to disruption of the *arcR* gene and not a second site mutation, the *arcR* gene was reintroduced into *S. gordonii* Δ*arcR* on a plasmid under regulation of the synthetic CP25 promoter to generate *S. gordonii* $arcR_{COMP}$. Biofilms formed by *S. gordonii* $arcR_{COMP}$ were very similar in structure to those of the wild-type. Quantitative assessment using image analysis software demonstrated that the biovolume of *S. gordonii* Δ*arcR* biofilms was significantly reduced compared with wild-type *S. gordonii* or the genetic complementation strain *S. gordonii* $arcR_{Comp}$, indicating that the biofilm formation defect observed in *S. gordonii* Δ*arcR* was a direct result of *arcR* gene disruption.

Analysis of the ArcR regulon

To assess the effects of disrupting the *arcR* gene on global gene expression in *S. gordonii*, microarrays were employed to analyse gene expression in *S. gordonii* DL1 or Δ*arcR* in high arginine or following a shift to no arginine. Cells were cultured to mid-exponential phase in arginine-replete

chemically defined growth medium, harvested and resuspended in high (0.5 mM) or no arginine. After 30 min, RNA was extracted and gene expression was monitored by microarray. Initially, the results from the microarray were validated by assessing the expression levels of 7 genes under high and no arginine using RT-qPCR. The genes *argC, argG, pyrA$_b$, arcA, arcB, arcD* and *amyB* (Figure 3) were selected for this analysis. Of these, *amyB* was was included as a control, and the other genes were chosen as they were previously reported to be implicated in arginine metabolism and transport.[19] The comparison showed a strong correlation between the microarray and RT-qPCR, with r^2 values of 0.995 and 0.777 for *S. gordonii* DL1 and Δ*arcR*, respectively, and slopes very close to 1 for each strain. To further assess the validity of individual microarray experiments, gene regulation from all *S. gordonii* DL1 and Δ*arcR* microarray samples under high and no arginine was compared by principle coordinates analysis (PCoA; data not shown). Four independent experiments were performed for each strain under each condition, giving a total of 16 microarray samples. However, PCoA identified two outliers in the data: one sample each of *S. gordonii* DL1 under high arginine and no arginine. Therefore these samples were removed from subsequent data analysis.

In total, 26 genes were significantly different between *S. gordonii* DL1 and Δ*arcR* under arginine-replete conditions (Table S3). This included *arcR* itself, which gave a detectable signal in the knockout strain even though the gene was not present. This signal was very low and was not dependent on arginine, and therefore was presumably due to background fluorescence. Of the other 25 genes, the most strongly regulated was a putative extracellular protein encoded by SGO_RS04150, which was up-regulated 110-fold in *S. gordonii* Δ*arcR* compared with the wild-type. The *argGH* operon (SGO_RS00865-00870) was up-regulated 5 to 6-fold. The gene *queA*, encoding a putative S-adenosylmethionine:tRNA ribosyltransferase-isomerase, was up-regulated 12-fold in *S. gordonii* Δ*arcR*. An apparent 4-gene operon (SGO_RS07910-07925) encoding a predicted magnesium transporter, a CAAX amino protease, a methyltransferase and peptide chain release factor 3, was up-regulated 4 to 6-fold. Two operons encoding phosphotranserase system (PTS) components were also up-regulated: *manLM* encoding mannose-specific enzyme IIAB and IIC was up-regulated ~2.5-fold and *levDEFG* encoding a fructose PTS enzyme IIA, IIB, IIC and Ia was up-regulated 7 to 12-fold. In addition, 3 hypothetical proteins, a possible pseudogene, an additional CAAX amino protease, peptidase S11, a putative ATP-dependent Clp protease ATP-binding protein, and a possible carboxylate-amine ligase were each up-regulated 2 to 4-fold. Only 3 genes were down-regulated in *S. gordonii* Δ*arcR*: SGO_RS02495 and SGO_RS08100 encoding putative PTS enzyme IIABC components (both down-regulated ~2-fold) and the *coiA* gene encoding a putative gene involved in genetic competence, which was 9-fold down-regulated.

To assess the impact of the *arcR* gene knockout on the ability of *S. gordonii* to respond to arginine, the ratio of gene expression following a shift to no arginine versus maintenance in high arginine for each of the above genes was calculated for *S. gordonii* DL1 and Δ*arcR* (Figure 4). This analysis revealed several different patterns of gene regulation. The two PTS operons *manLM* and *levDEFG* along with the 4-gene operon containing a putative magnesium transporter (SGO_RS07910-07925) had little or no arginine-dependent regulation in *S. gordonii* DL1 but were more strongly down-regulated in response to arginine restriction in *S. gordonii* Δ*arcR*. The reverse was seen with

SGO_RS07765 encoding peptidase S11 and *coiA*, which were more strongly down-regulated in the wild-type than *S. gordonii* Δ*arcR* in response to the removal of arginine. The *queA* gene, SGO_RS00730 (hypothetical protein) and SGO_RS09090 (Clp protease ATP binding protein) were up-regulated in the absence of arginine to approximately the same extent in the wild-type and Δ*arcR* mutant. SGO_RS02495 glucose PTS enzyme IIABC subunit, SGO_RS01240 encoding a CAAX amino protease, SGO_RS08100 encoding trehalose PTS enzyme IIABC and SGO_RS10240 carboxylate-amine ligase were expressed at similar levels in no versus high arginine the wild-type, but were up-regulated under arginine restriction in *S. gordonii* Δ*arcR*. Finally, SGO_RS07655 and SGO_RS04295 encoding hypothetical proteins, the pseudogene SGO_RS06275 and *argGH* were strongly down-regulated under high arginine in the wild-type and less strongly down-regulated in *S. gordonii* Δ*arcR*.

Gene regulation in S. gordonii *Δ*argR *and Δ*ahrC

To compare the ArcR regulon with those of the orthologous ArgR-family regulators, ArgR and AhrC, microarray analysis was performed on the *S. gordonii* Δ*argR* and Δ*ahrC* mutants. The validity of the microarrays was checked by RT-qPCR for the genes *argC, argG, pyrA$_b$, arcA, arcB, arcD* and *amyB* (Figure 3). As with the previous microarrays, there were strong correlations between the microarray and RT-qPCR data (r^2 values >0.95 and slopes close to 1). There were no significant differences between gene expression in *S. gordonii* Δ*argR* or Δ*ahrC* under either high or no arginine. The microarrays for *S. gordonii* Δ*argR* and Δ*ahrC* were performed by a different operator and at a different time from the *S. gordonii* DL1 and Δ*arcR* arrays. Performing microarray analyses at different times can lead to 'batch effects' where absolute gene expression levels are slightly different across arrays due to the use of different reagents or slight changes in protocol.[30] Further, a relatively high proportion of probes (~19%) did not give a quantifiable signal from the *S. gordonii* Δ*argR* and Δ*ahrC* samples. To assess the relationships between the microarrays, the data from each array was compared by PCoA (Figure S1). The data for *S. gordonii* Δ*argR* and Δ*ahrC* clustered together and were well separated from *S. gordonii* DL1 and Δ*arcR*. Although it is possible that these observations reflect genuine biological differences between the strains, the comparisons between *S. gordonii* Δ*argR*/Δ*ahrC* and *S. gordonii* DL1/*arcR* must be treated with caution. Therefore, it was not possible to define the ArgR and AhrC regulons by direct comparison with *S. gordonii* DL1 under high or no arginine. Instead, rank product analysis was used to identify significant differences in gene expression between *S. gordonii* Δ*argR*/Δ*ahrC* and *S. gordonii* DL1 under high arginine.

Rank product analysis does not quantify the differences in expression levels between mutants and wild-type, but instead ranks the most strongly regulated genes. In total, 109 genes were identified using this approach that were differentially regulated in *S. gordonii* Δ*argR* and/or Δ*ahrC* compared with *S. gordonii* DL1 (Table S4). The most strongly up-regulated genes were the *argCJBD* arginine biosynthesis operon, consistent with the previously identified roles of ArgR and AhrC as arginine-dependent repressors of transcription of arginine biosynthesis genes. Another arginine biosynthesis gene operon, *pyrRA$_a$A$_b$*, and the histidine biosynthesis operon (SGO_RS06875-SGO_RS06920) were also strongly and co-ordinately up-regulated in the mutants. Approximately as many genes were down-regulated in the mutants as up-regulated. Down-regulated genes included *purE* and *purM*, involved in purine metabolism, and *scaC* and *scaA* encoding components of an ABC-type manganese transporter.

Of the 26 genes identified as ArcR-regulated, 10 genes were not detected in the *S. gordonii* Δ*argR* and Δ*ahrC* samples (shaded grey in Figure 4). The *arcR* and *queA* genes appeared slightly more strongly up-regulated in arginine restriction in *S. gordonii* Δ*argR* and Δ*ahrC* mutants than the wild-type (Figure 4). The SGO_RS07910-07925 operon and *manLM* were weakly down-regulated in no arginine in *S. gordonii* Δ*argR* and Δ*ahrC*. This pattern of gene expression was more similar to *S. gordonii* DL1 than to *S. gordonii* Δ*arcR*. By contrast, the pattern of arginine-dependent gene regulation in *S. gordonii* Δ*argR* and Δ*ahrC* for the S11 peptidase gene, SGO_RS07655 and SGO_RS04295 hypothetical protein-encoding genes and *argGH* was more similar to *S. gordonii* Δ*arcR* than to the wild-type. SGO_RS09090 Clp protease ATP binding protein locus was up-regulated in no arginine to similar extents in all *S. gordonii* strains. Overall, these data demonstrate that disruption of *arcR* leads to several unique changes in gene expression that are not seen in *S. gordonii* Δ*argR* or Δ*ahrC*.

Role of SGO_RS04150 in biofilm formation

Of the differentially regulated genes, SGO_RS04150 (SGO_0846) was of particular interest since it was strongly regulated in *S. gordonii* Δ*arcR*. This gene is annotated as encoding a cell wall protein in the NCBI database. Using SignalP,[31] we identified a putative N-terminal secretion signal. However, using PSORTb[32] we were unable to detect an LPxTG cell wall anchor or a lipoprotein motif and it is possible that the protein is secreted from the cell surface. To confirm that this gene is regulated by ArcR, we assessed expression levels by RT-qPCR under arginine-replete or no arginine conditions (Figure Xa). Under high arginine, expression was significantly increased 17-fold in *S. gordonii* Δ*arcR* compared with wild-type. In line with the microarray data (Figure 5a), expression was slightly elevated in no arginine in *S. gordonii* DL1 and slightly decreased in *S. gordonii* Δ*arcR*. In view of the strong regulation by ArcR, we have termed the SGO_RS04150 gene '*earA*', encoding '**E**xtracellular **A**rcR-**R**egulated protein A'. To assess the role of *earA* in *S. gordonii* biofilm formation, the gene was disrupted in the wild-type and the Δ*arcR* background. Using crystal violet staining, biofilm formation by *S. gordonii* Δ*earA* was slightly reduced compared with the wild-type (Figure 5b) but the difference was not significant. Similarly, knockout of *earA* in the Δ*arcR* or $arcR_{COMP}$ backgrounds did not significantly reduce biofilm formation. Therefore, it appears that dysregulation of *earA* expression is not the major cause of the biofilm defect observed in *S. gordonii* Δ*arcR*.

DISCUSSION

Our studies identified ArcR as a key determinant of biofilm formation in *S. gordonii*. As no arginine metabolic gene disruptions affected biofilm formation, it appears that ArcR may be acting through a mechanism that is independent of regulation of arginine metabolism. Interestingly, a different ArgR family regulator, EF0676, the orthologue of ArgR, was identified in a transposon mutant screen for biofilm-associated genes in *Enterococcus faecalis.*[33] Subsequently, the homologue of AhrC, EF0983, was also implicated in biofilm formation since strains carrying a transposon insertion were impaired in the development of biofilms *in vitro* and in biofilm infection models of infective endocarditis and catheter-associated urinary tract infection.[34] The *E. faecalis* genome also contains two genes encoding orthologues of *S. gordonii* ArcR that have been named *argR2* and *argR1*.[35] However, to the

best of our knowledge neither of these, or any other orthologues of *S. gordonii arcR* have yet been associated with biofilm formation phenotypes.

ArgR-family regulators form hexameric complexes that are activated by the binding of arginine, and bind to regulatory regions termed ARG box elements in the upstream region of target genes.[36] It is not clear why streptococci and related bacteria produce multiple ArgR family homologues. In particular, the function of ArgR and AhrC is often very similar, and knocking out one or other gene is sufficient to abrogate arginine-dependent regulation of target genes.[19] It has been postulated that these may form a heterohexameric complex or that they may co-ordinate gene expression by cross-regulating one another.[37] Transcriptome analysis in *Lactococcus lactis* or *Streptococcus pneumoniae* has identified minor differences between the ArgR (termed ArgR1 in *S. pneumoniae*) and AhrC regulons, indicating that heterohexameric complexes containing both ArgR and AhrC are not essential for gene regulation.[38,39] By contrast, direct comparison between gene expression in *S. gordonii* Δ*argR* and Δ*ahrC* here did not identify any significant differences between the strains in either high or no arginine. Nearly 20% of the probes failed to make the quality control thresholds and it is possible that some differences between the ArgR and AhrC regulons were missed in our analysis. Nevertheless, it appears that ArgR and AhrC have very similar functions in *S. gordonii*.

In a previous microarray analysis of *S. gordonii* DL1, >450 genes were shown to be significantly regulated following a shift from high to no arginine.[19] *S. gordonii* has the capacity to biosynthesise arginine, but does not grow aerobically in media lacking arginine.[21] Therefore, it is possible that some of this gene regulation was an adaptation to stress rather than a specific response to the lack of arginine. In growth experiments using FMC, *S. gordonii* Δ*argR*, Δ*ahrC* and Δ*arcR* were similarly impaired in aerobic growth in the absence of arginine (data not shown). Therefore, differences in gene regulation between *S. gordonii* DL1 and the mutant strains are likely to reflect the function of the regulators rather than simply stress imposed by the lack of arginine and the subsequent growth arrest. Many of the genes that were regulated in *S. gordonii* DL1 following a shift from high to no arginine, including arginine and histidine biosynthesis operons, were up-regulated specifically in high arginine in *S. gordonii* Δ*argR* or Δ*ahrC* compared with wild-type (Table S4). This pattern of regulation is consistent with the action of ArgR/AhrC acting as repressors in arginine-bound form. Using the transcription factor binding site prediction programme PePPER [40] to search for *L. lactis* ARG box elements throughout the *S. gordonii* genome, 6 ARG boxes were identified upstream of genes that were up-regulated in *S. gordonii* Δ*argR* or Δ*ahrC*. These were upstream of *argR*, *argC* (two elements), *pyrR*, *acnA* and SGO_RS08115. Of these, *argR* encodes the ArgR regulator, *argC* is the first gene in the *argCJBD* arginine biosynthesis operon and *pyrR* is the first gene in the *pyrRA*$_a$*A*$_b$ arginine biosynthesis operon. On the other hand, *acnA*, encoding aconitate hydratase and SGO_RS08115, encoding phosphoenolpyruvate carboxykinase, are involved in the citric acid cycle, indicating that arginine sensing is linked to central metabolism in *S. gordonii*. Although ArgR/AhrC are thought to act primarily as repressors of transcription, it is possible that they may also be activators in some conditions. The above screen identified one ARG box element upstream of *scaC*, the first gene in the *scaCBA* operon encoding an ATP-binding cassette manganese transporter,[41] that was down-regulated in *S. gordonii* Δ*argR* and Δ*ahrC* under high or low arginine. However, *scaB* was not

regulated and *scaA* was only regulated in *S. gordonii* Δ*argR* under high arginine. Therefore, it is not clear what impact arginine-sensing has on the expression or function of the transporter as a whole. It is likely that there are many other ARG box elements upstream of ArgR/AhrC-regulated genes in *S. gordonii*. In *E. coli*, 62 ArgR binding regions have been identified by chromatin immunoprecipitation (ChIP-Seq).[36,42] Measurement of ArgR/AhrC interactions with DNA in promoter regions by ChIP-Seq, electrophoretic mobility shift or DNase I footprinting would be required to determine which genes are directly regulated by ArgR/AhrC in *S. gordonii*.

The target DNA binding sequence for *S. gordonii* ArcR has been investigated by DNase I footprinting, and shown to consist of a 27 bp region with little identity to ARG box motifs [43]. ArcR has previously been shown to regulate the expression of the *arcABC* operon, *queA* and *argGH.*[19,44,45] The microarray analysis identified *queA* and *argGH* as genes that were up-regulated in *S. gordonii* Δ*arcR* compared with wild-type under high arginine. Interestingly, however, *arcA*, *arcB* and *arcC* were not differentially expressed between *S. gordonii* DL1 and Δ*arcR* under high or no arginine by microarray analysis. All three genes were up-regulated approximately 3 to 4-fold in high arginine compared with no arginine in both strains. It is well-established that disruption of the *arcR* gene abrogates arginine-dependent activation of *arcABC* expression[19,45] and it is therefore not clear why this was not seen in the microarray data. However, using RT-qPCR which has a higher dynamic range than microarrays,[46] it was found that expression of *arcA* and *arcB* was significantly lower in *S. gordonii* Δ*arcR* than the wild-type under high arginine, in line with previous observations (data not shown).

In addition to known genes, ArcR was shown to have a major impact on the regulation of phosphoenolpyruvate:sugar phosphotransferase system (PTS) components. Oral streptococci utilise many different carbohydrates for growth and PTSs are the primary mechanism for sugar uptake.[47] Using proteomic analysis, it was found that PTSs are a common target for gene regulation in *S. gordonii* following interspecies interactions with *P. gingivalis* and *F. nucleatum.*[48] In addition, the PTS operon *fruRBA* was up-regulated following adhesion of *S. gordonii* to *C. albicans.*[20] This operon was previously shown to have a key role in biofilm formation.[49] Although *fruRBA* was not regulated by ArcR, another PTS (*levDEFG*) was strongly (7 to 12-fold) up-regulated in *S. gordonii* Δ*arcR* under high arginine, indicating that ArcR represses expression of this operon when arginine is available. The genes *levDEFG* encode enzyme IA, IIA, IIB and IIC components of a PTS that primarily transports fructose.[50] It is not yet known whether this system is required for the development of biofilms by *S. gordonii*. Similarly, the PTS enzyme IIAB and IIC components encoded by *manLM* have been assessed for their roles in sugar uptake and found to transport glucose, galactose and mannose, but have not been assessed for biofilm-associated functions.[50] Three additional PTSs have been shown to be important for biofilm formation by *S. gordonii.*[51] Of these, the *bfb* locus encoding a cellobiose PTS has previously been shown to be regulated by arginine and by coaggregation with *A. oris*.[19,21] Although the *bfb* genes were not significantly regulated in *S. gordonii* Δ*arcR* compared with wild-type in our microarray analyses, there was a trend towards up-regulation under high arginine. Therefore, we assessed the expression of *bfbC* and *bfbF* in *S. gordonii* DL1 and Δ*arcR* by RT-qPCR. Both genes were approximately 4-fold increased in *S. gordonii* Δ*arcR* compared with wild-type under high arginine, and the difference was statistically significant ($p<0.05$) in the case of *bfbC*. Therefore,

it is possible that dysregulation of the *bfb* locus in the absence of ArcR may be partly responsible for the observed biofilm defects of *S. gordonii* Δ*arcR*.

In addition to PTSs, disruption of *arcR* also led to a change in the regulation of an apparent 4-gene operon containing a magnesium transporter, CAAX amino protease, methyltransferase and peptide chain release factor 3. These genes were only weakly regulated by arginine in *S. gordonii* DL1, but were more strongly down-regulated in by a shift to no arginine in *S. gordonii* Δ*arcR*. It is not clear why these genes are linked to arginine sensing in *S. gordonii*. However, it is noteworthy that this operon is immediately upstream of *acnA*, which contains an ARG box element in the promoter region. The gene encoding a protease was one of two CAAX protease-encoding genes that were regulated by ArcR. The function of these proteins is not fully understood, but some of them play a role in self-immunity against bacteriocins.[52] Oral streptococci possess many paralogues of genes encoding CAAX amino proteases: *S. gordonii* has 14, whereas *S. sanguinis* has 21 paralogues, more than any other species that has been analysed to date.[52] One of these proteases in *S. gordonii* (BfrH) and two in *S. sanguinis* (BfrH1 and BfrH2) are regulated by biofilm-associated BfrAB two-component systems and it has been speculated that these may play important roles in processing proteins that are secreted in order to promote biofilm development.[53] The ClpP protease complex may also be involved in controlling the quality and integrity of secreted proteins involved in biofilms. Thus, an ATP binding protein associated with the ClpP complex was up-regulated in *S. gordonii* Δ*arcR* compared with wild-type under high arginine. The ClpP complex consists of ClpP protease and a number of ATPases that together are required for biofilm formation in *Streptococcus mutans.*[54] Disruption of the *arcR* gene also led to a change in expression of the competence protein CoiA. In *S. pneumoniae* CoiA is involved in genetic recombination and is only transiently expressed during competence. It is possible that disruption of *arcR* led to the initiation of competence development,[55] although it is important to note that other competence genes were not differentially regulated between *S. gordonii* DL1 and Δ*arcR*. The gene encoding carboxylate-amine ligase is immediately upstream of *purE* involved in purine biosynthesis, and may even be part of the same operon. The *purE* gene was down-regulated in *S. gordonii* Δ*argR* and Δ*ahrC* compared with wild-type under high and no arginine. Therefore, it appears that all three ArgR family regulators may co-ordinate to control this locus, as they do for the regulation of *argGH*. Finally, peptidase S11 is a D-alanyl-d-alanine carboxypeptidase, involved in peptidoglycan biosynthesis. It is not clear whether this or any of the hypothetical proteins that were regulated by ArcR could be involved in biofilm formation.

The strongest regulation in *S. gordonii* Δ*arcR* was a gene of unknown function, which we have termed *earA*. This gene was up-regulated in *S. gordonii* following a shift to no arginine. However, in *S. gordonii* Δ*arcR* expression of *earA* was strongly (>100-fold in the microarray analysis) up-regulated under high arginine. The function of EarA is unknown. It is predicted to have an SCP-like extracellular protein domain; the function of these domains is not well-understood.[56] It was possible that the dramatic increase in expression of *earA* in *S. gordonii* Δ*arcR* may be associated with the observed biofilm defect. However, disruption of the *earA* gene in *S. gordonii* DL1, Δ*arcR* or $arcR_{Comp}$ did not affect biofilm formation compared with the respective parent strain, indicating that EarA is not a major contributor to biofilm development.

In conclusion, have identified ArcR as a key gene in biofilm formation by *S. gordonii*. The mechanism by which ArcR affects biofilm growth or stability remains unclear, but it is possible that changes in the co-ordination of PTS expression may be at least partly responsible. Further work is required to establish whether arginine is the only stimulus for ArcR, and to determine how arginine itself modulates *S. gordonii* biofilm development through activation of the ArcR regulatory pathway. In addition, assessment of the role of ArcR in the early stages of biofilm formation, or in biofilm formation under fluid flow, may help to shed light on the specific function of ArcR in biofilm development by *S. gordonii*.

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FIGURE LEGENDS

Figure 1. Biofilm formation by *S. gordonii* DL1 and isogenic mutants disrupted in arginine metabolism or regulation genes. Biofilms were grown aerobically in TYEG medium for 18 h, and quantified by measuring absorbance of crystal violet-stained cells at 562 nm. Biofilm formation by Δ*arcR* single or double mutant strains was significantly lower than those of the other mutant strains ($p<0.05$; indicated by asterisks). Bars represent arithmetic means of three independent biological repeats, and standard error is shown. All *S. gordonii* Δ*arcR* mutant strains show a significant biofilm defective phenotype, not displayed by the other mutants tested here.

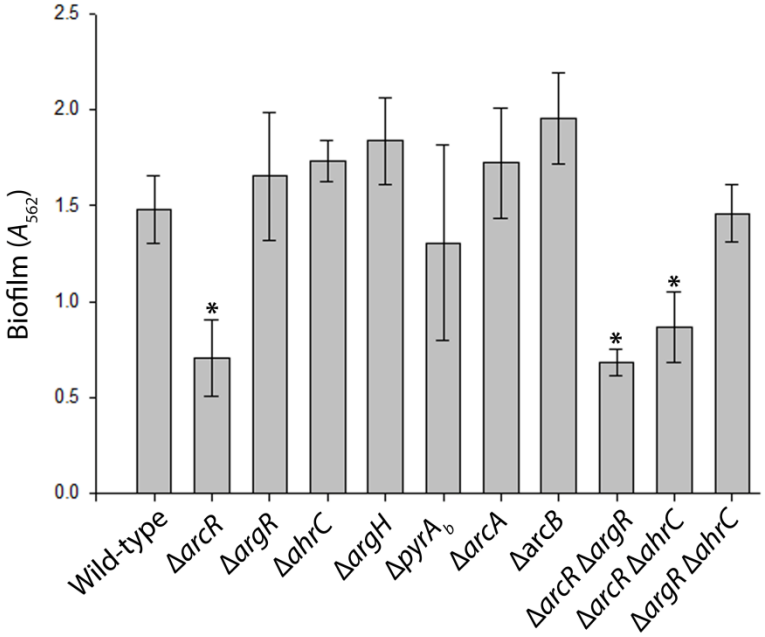
Figure 2. Visualisation of biofilms formed by *S. gordonii* DL1, Δ*arcR* and *arcRComp*. Biofilms were grown in TYEG medium aerobically for 18 h, stained with BacLight LIVE:DEAD stain and visualised by confocal laser scanning microscopy. 3D renderings (left) or maximum projection images (right) are shown. Live cells stained with Syto 9 appear green; red staining shows compromised cells that have taken up propidium iodide. Images were quantified using Volocity software. The quantification (mean ± standard errors) of biovolume is shown for 3 independent experiments. Biovolumes of biofilms from all strains were significantly different from one another.

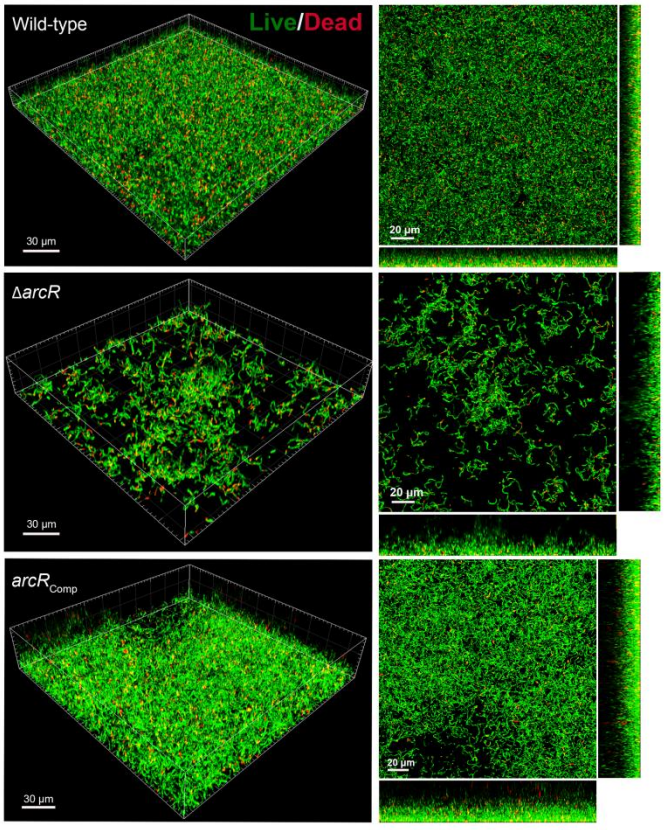
Figure 3. Validation of the *S. gordonii* DL1, Δ*arcR,* Δ*argR* and Δ*ahrC* microarrays by comparison with RT-qPCR data for each strain. Gene expression was measured as a $\log_2$ fold-change in no arginine compared with high arginine in *S. gordonii* DL1, Δ*arcR,* Δ*argR* and Δ*ahrC* strains. The linear regression line for each correlation is shown. Values on the graphs indicate the r^2 value and the slope of the line.

Figure 4. Heat map showing differential gene regulation in *S. gordonii* DL1, Δ*arcR*, Δ*argR* and Δ*ahrC* mutants in response to arginine. Microarray analysis was carried out on cDNA samples

from *S. gordonii* strains during a shift to no arginine versus maintenance in high arginine. Colour coding represents fold change in no arginine compared with high arginine, and negative values (blue tones) indicate down-regulation. Data from *S. gordonii* DL1 and Δ*arcR* strains are not directly comparable with those from *S. gordonii* Δ*argR* and Δ*ahrC* due to concerns about batch effects between the microarray experiments, which were conducted at different times. Gray areas in *S. gordonii* Δ*argR* and Δ*ahrC* microarrays show genes that did not pass the quality threshold, and for which no reading was obtained. *QueA is a predicted S-adenosylmethionine:tRNA ribosyltransferase-isomerase.

Figure 5. Expression of *earA* and impact of *earA* gene disruption on biofilm formation by *S. gordonii*. (a) RT-qPCR analysis of *earA* expression in *S. gordonii* DL1 and Δ*arcR* under high and no arginine. Cells were grown in FMC medium, harvested and resuspended in FMC without arginine or arginine-replete FMC. Following incubation for 30 min, gene expression was measured by RT-qPCR and is expressed as $\log_2$ fold-change values. White bars represent the *earA* expression in *S. gordonii* DL1 and black bars represent *earA* expression in *S. gordonii* Δ*arcR*. Bars represent means of three independent biological replicates, and standard error is shown. (b) Impact of *earA* gene disruption on biofilm formation. Biofilms were grown in TYEG medium aerobically for 18 h, and quantified by measuring absorbance of crystal violet-stained cells at 562 nm. Bars represent means of three independent biological replicates, and error bars show standard error. Significant differences between mutants and wild-type are indicated by an asterisk. Disruption of *earA* did not significantly reduce biofilm formation in wild-type or Δ*arcR* genetic backgrounds.





	Wild-type	Δ*arcR*	*arcR*$_{Comp}$
Biovolume (µm³/µm²)	0.80 ± 0.08	0.26 ± 0.02	1.67 ± 0.12